

REMARKS

Entry of the foregoing amendments is respectfully requested.

Summary of Amendments

Upon entry of the foregoing amendments, claims 1-16 are canceled and claims 17-36 are added, whereby claims 17-36 will be pending, with claims 17 and 26 being independent claims. Support for the new claims can be found throughout the present specification and in the canceled claims. In particular, claims 1-16 have been rewritten as method claims.

Applicants emphasize that the cancellation of claims 1-16 is without prejudice or disclaimer, and Applicants expressly reserve the right to prosecute these claims in one or more continuation and/or divisional applications.

Summary of Office Action

As an initial matter, Applicants note with appreciation that the Office Action indicates that the claim for priority is acknowledged and that certified copies of the priority documents have been received.

Applicants further note with appreciation that the Examiner has indicated consideration of Information Disclosure Statement filed August 12, 2005 by returning a signed and initialed copy of the Form PTO-1449 submitted therein.

The present Office Action alleges that Applicants have failed to comply with one or more conditions for receiving the benefit of a prior-filed application under 35 U.S.C. § 119(e), 120, 121 or 365(c).

The present Office Action further alleges that the executed declaration filed together with the present application does not acknowledge the filing of any foreign application.

Claims 1-16 are rejected under 35 U.S.C. § 101 because the claimed recitation of a use or an intended use, without setting forth any steps involved in the process, allegedly results in an improper definition of a process.

Claims 5-8 and 10-16 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for a method of treating allergic diseases, allegedly does not reasonably provide enablement for the prevention of all allergic diseases.

Claims 1-16 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite.

Claims 1-16 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by US Patent Application No. 2002/0010159 to Weigle et al. (hereafter "WEIGELE").

Response to Office Action

Withdrawal of the rejections of record is respectfully requested, in view of the foregoing amendments and the following remarks.

Response to Alleged Non-Compliance With the Conditions For Receiving The Benefit of an Earlier Filing Date

At pages 2 to 4 under the heading "Priority" the present Office Action alleges that Applicants have failed to comply with one or more conditions for receiving the benefit of a prior-filed application under 35 U.S.C. § 119(e), 120, 121 or 365(c).

From the Examiner's comments it appears that there may be a misunderstanding. In particular, the present application does not claim the benefit under 35 U.S.C. § 120 of any prior filed U.S. application (including any U.S. application which is a National Stage of an International Application filed under the Patent Cooperation Treaty), but is itself the National Stage of an International Application, i.e., there is only one U.S. application. As such, the present application is entitled to the filing date of the International Application of which it is the National Stage without the need to make a specific reference to the International Application. In other words, the present application is neither a continuation (or continuation-in-part) nor a divisional application of any prior U.S. application.

In view of the foregoing, Applicants respectfully submit that the present application is entitled to a U.S. filing date of November 3, 2003 (i.e., the filing date of International Application PCT/KR2003/002332).

Response to Alleged Non-Compliance with the Requirements of 37 C.F.R. 1.63(c)

At page 4 of the present Office Action it is alleged that the present executed declaration does not acknowledge the filing of any foreign application.

Applicants respectfully submit that this allegation is obviously incorrect. The two foreign (Korean) priority applications of the present National Stage application (10-2002-0067653 filed November 2, 2002, and 10-2003-0075511, filed October 28, 2003) are identified at the bottom of page 1 of the executed declaration filed together with the present application, where it is also indicated that the priority of these applications is claimed.

In view of the foregoing, Applicants submit that the requirements of 37 C.F.R. 1.63(c) have clearly been complied with.

Response to Rejection of Claims under 35 U.S.C. § 101

Claims 1-16 are rejected under 35 U.S.C. § 101 because the claimed recitation of a use or an intended use, without setting forth any steps involved in the process, allegedly results in an improper definition of a process.

Applicant respectfully submit that canceled claims 1-16 are composition claims, i.e., not method claims. At any rate, claims 1-16 have been rewritten as method of use claims, submitted herewith, thereby rendering the rejection under 35 U.S.C. § 101 moot.

Response to Rejection of Claims under 35 U.S.C. § 112, First Paragraph

Claims 5-8 and 10-16 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for a method of treating allergic diseases, allegedly does not reasonably provide enablement for the prevention of all allergic diseases. In particular, the Examiner appears to request experimental evidence supporting the

contention that the active ingredients specified in the rejected claims can actually prevent allergic diseases by administering the specified active ingredients.

This rejection is respectfully traversed. Applicants initially note that the rejected claims are not drawn to the prevention of allergic diseases in general, but to the prevention of allergic diseases caused by HRF.

Further, the present Office Action does not explain why despite the experimental results set forth in the present specification, it is not credible that the specified active ingredients are capable of preventing allergic diseases caused by HRF.

In this regard, Applicants note that HRF plays an important role in allergic reactions by inducing secretion of the various cytokines. For instance, it has already been reported that when treating basophils with HRF, the secretion of histamine which is important in allergic symptom development increases rapidly (see the enclosed article by J. T. Schroeder et al., J. Immunol. 1997, 159: 447-452), and that when treating eosinophils with HRF, the secretion of IL-8 cytokine is promoted (see the enclosed article by R. Bheeka-Escura, Blood 2000, 96: 2191-2198)¹.

Based on the above-mentioned facts it can be anticipated that if proton pump inhibitors are administered to an allergy patient after contacting with an allergen, an allergy treating effect can be achieved because there is an influence only by already secreted HRF, without any further secretion of HRF.

¹ In accordance with M.P.E.P. § 609C(3), the documents cited above in support of Applicants' remarks are being submitted as evidence directed to an issue raised in the mentioned Official Action, and no additional fee or Certification pursuant to 37 C.F.R. §§ 1.97 and 1.98, or citation on a FORM PTO-1449 is believed to be necessary.

If proton pump inhibitors are administered to an allergy patient before contacting with an allergen, an allergy preventing effect can be achieved since the cytokines that take part in an allergic reaction or immunocyte reaction decline due to an advance interruption of the HRF secretion.

Even though there are continuous influences by other allergic reaction factors, allergic symptoms are significantly diminished because of the restrained action of HRF that plays an important role in allergic reactions.

The above anticipation is confirmed by the experimental results described in the present application. Specifically, in Example 2 (2) at pages 12 and 13 of the present application it is described that the mortality rate of mice treated with the anaphylaxis-causing compound 48/80 could be significantly decreased by administering to the mice pantoprazole 30 minutes before the administration of compound 48/80. This is clearly indicative of an anaphylaxis-preventing effect.

Qualitatively similar results are described in Example 3 at pages 15-17 of the present application regarding the preventive effect of pantoprazole with respect to allergic rhinitis. Again, the pantoprazole was administered 30 minutes before the rhinitis was induced.

It is submitted that for at least the foregoing reasons the rejection of claims 5-8 and 10-16 under 35 U.S.C. § 112, first paragraph, is unwarranted, wherefore withdrawal thereof is respectfully requested.

Response to Rejection of Claims under 35 U.S.C. § 112, Second Paragraph

Claims 1-16 are rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. In this regard, the Office Action alleges that it is not clear what method/process is to be encompassed for inhibiting a secretion or the use for the treatment of allergic diseases.

Applicants respectfully submit that the present independent claims are generally drawn to methods of inhibiting the secretion of IgE-dependent histamine-releasing factor (HRF) in a patient, which methods comprise an administration to the patient of an effective amount of a compound having proton pump inhibitor activity. In view thereof, it is respectfully submitted that the rejection of the claims under 35 U.S.C. § 112, second paragraph, is rendered moot.

Response to Rejection of Claims under 35 U.S.C. § 102(b)

Claims 1-16 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by WEIGELE. In this regard, the rejection essentially asserts that WEIGELE discloses compositions which contain a benzimidazole proton pump inhibitor such as omeprazole as active ingredient. The rejection concedes that the cited document does not disclose the use of the benzimidazole proton pump inhibitors described therein for inhibiting the secretion of HRF, but essentially states that a new intended use does not make an otherwise known composition new.

Applicants respectfully submit that claims 17-36 submitted herewith are drawn to methods of inhibiting the secretion of IgE-dependent histamine-releasing factor (HRF) in

a patient. As already acknowledged in the present Office Action, WEIGELE does not teach the use of the proton pump inhibitors disclosed therein for inhibiting the secretion of HRF. For this reason alone, the present claims are not anticipated by WEIGELE. Accordingly, withdrawal of the rejection of the present claims under 35 U.S.C. § 102(b) is warranted and respectfully requested.

CONCLUSION

In view of the foregoing, it is believed that all of the claims in this application are in condition for allowance, which action is respectfully requested. If any issues yet remain which can be resolved by a telephone conference, the Examiner is respectfully invited to contact the undersigned at the telephone number below.

Respectfully Submitted,
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Recombinant Histamine-Releasing Factor Enhances IgE-dependent IL-4 and IL-13 Secretion by Human Basophils¹

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Human recombinant histamine-releasing factor (HrHRF) is known to directly stimulate histamine release and IL-4 secretion from basophils of selected atopic donors in a reaction requiring the expression of a particular type of IgE, referred to as IgE⁺. In this study, HrHRF is shown to affect the IgE-mediated release of IL-4, IL-13, and histamine from basophils not normally releasing to this protein (i.e., those expressing IgE⁻). Priming with several different concentrations of HrHRF for 15 min enhanced basophil secretion of IL-4 and histamine after 4 h in a dose-dependent fashion following activation with anti-IgE Ab (10 ng/ml). This effect of HrHRF priming also occurred in cultures activated with 1 or 100 ng/ml of anti-IgE Ab. The secretion of IL-13 protein was enhanced similarly by HrHRF priming in cultures stimulated for 16 to 20 h with anti-IgE Ab. There were, however, no apparent changes in the secretion of histamine or cytokine by basophils primed with HrHRF and activated with the IgE-independent secretagogue, FMLP. These findings suggest that HrHRF modifies the response of basophils for IgE-dependent secretion by binding to a specific receptor, broadening the possible role of this protein in chronic allergic inflammation. *The Journal of Immunology*, 1997, 159: 447-452.

There is increasing evidence to support the importance of basophils at sites of allergic inflammation. Basophils were shown to be present in biopsies of asthmatic patients who had airway narrowing (1). Furthermore, an increase of basophils was noted in the lungs of patients dying of asthma, and their presence correlated with bronchial hyper-responsiveness in patients experiencing asthmatic episodes (2, 3). While it is well known that basophils release inflammatory mediators, such as histamine and LTC₄, recent studies show that they also generate and secrete several cytokines including IL-4 (4-7), IL-13 (8-10), and macrophage inflammatory protein-1 α (11). Most important, a striking correlation was found between IL-4 mRNA and protein and the presence of basophils, suggesting that these cells are a major source of this cytokine (6). While this was shown using a basophil-specific stimulus (i.e., anti-IgE), a more recent study has confirmed these findings by showing that basophils are the major source of IL-4 even in cultures stimulated with Ag (12). The production of cytokines by basophils is strong evidence that these cells modulate the immune responses of other cell types participating in allergic lesions. For example, in vitro studies have shown that IL-4 and IL-13 mediate several important activities occurring in allergic inflammation including IgE isotype switching in B cells (13, 14) and in increasing the expression of vascular adhesion molecules important for eosinophil and basophil endothelial transmigration (15-17). In addition, IL-4, but not IL-13, has been shown to act directly on T lymphocytes to induce their development into cells express-

ing a Th2 phenotype (18). Finally, both cytokines have been found at sites of allergic inflammation in the lung (19, 20).

We have described previously a histamine-releasing factor (HRF),³ found in lavage fluids obtained from allergic lesions, which caused the release of histamine and LTC₄ from a subpopulation of allergic donors (21). We proposed that this HRF produced secretion by interacting with a subpopulation of IgE molecules that we termed IgE⁺. By definition, the basophils expressing IgE⁺ on their surface did not support mediator release when stimulated with HRF. This factor has recently been subcloned and expressed. Human recombinant HRF (HrHRF) shows no molecular similarities to any known IL, chemokine, or Ag (22). In addition to causing histamine release from basophils expressing IgE⁺, HrHRF was subsequently found to stimulate the generation and secretion of IL-4 (23). Interestingly, the time course necessary for HrHRF to induce IL-4 secretion from basophils, and the levels of cytokine secreted, were identical with those induced by anti-IgE Ab.

The mechanisms by which HrHRF exerts its activity on human basophils are presently unknown. The fact that HrHRF causes histamine release and IL-4 secretion only from cells expressing IgE⁺ is consistent with the belief that this protein mediates activity by interacting directly with this unique form of Ig. However, several attempts to show that HrHRF interacts with soluble IgE, by ELISA or affinity chromatography, have been unsuccessful. Furthermore, we have shown recently that HrHRF can modulate the function of cells not expressing IgE⁺. Preincubation, or priming, with this protein enhances histamine release induced by anti-IgE Ab or Ag (24). In the present study, we addressed whether the secretion of IL-4 and IL-13 protein by basophils is affected similarly by HrHRF priming by testing the ability of this protein to act on cells expressing IgE⁻ and not IgE⁺. Our results show that HrHRF also enhances the secretion of IL-4 and IL-13 protein by IgE-bearing basophils activated by anti-IgE Ab or Ag. The fact that HrHRF modulates IgE-mediated histamine release, IL-4, and IL-13 protein

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³ Abbreviations used in this paper: HRF, histamine-releasing factor; C-IMDM, conditioned Iscoves' modified Dulbecco's medium; HrHRF, human recombinant histamine-releasing factor; PAC, piperazine-N,N'-bis(2-ethane sulfonic acid)/albumin/glucose; TMB, 3,3',5,5'-tetramethylbenzidine.

secretion by basophils prepared from normal donors, while directly causing the release of these products from cells bearing IgE⁺, supports the belief that this protein may bind a specific receptor on the cell surface. Furthermore, this finding suggests that HrHRF represents a unique cytokine, having an important role in modulating the release of inflammatory mediators and cytokines by basophils and possibly other cell types that participate in allergic disease.

Materials and Methods

Buffers and media

PIPES (10X) contained 250 mM PIPES, 1.10 M NaCl, and 50 mM KCl, pH 7.4. A PIPES/albumin/glucose buffer (PAG) contained one-tenth 10X PIPES in addition to 0.003% human serum albumin (Calbiochem-Bering Corp., La Jolla, CA) and 0.1% d-glucose. PAG-EDTA additionally contained 4 mM EDTA. Isotonic Percoll (referred to in this work as 100% Percoll) consisted of 1 part 10X PIPES and 9 parts Percoll (Pharmacia, Piscataway, NJ). Percoll solutions of 55% ($d = 1.072$) and 63% ($d = 1.082$) were made by mixing the appropriate amounts of 1X PIPES and 100% Percoll. Conditioned IMDM (C-IMDM) consisted of Iscoves' modified Dulbecco's medium containing 5% heat-inactivated (56°C for 30 min) FBS (Sigma Chemical Co., St. Louis, MO), 1X nonessential amino acids, and 5 µg/ml gentamicin. Assay buffer for the in-house ELISA consisted of a phosphate buffer containing Tween-80 and 1% BSA (made by adding 0.1 M phosphate monobasic to 0.1 M phosphate dibasic, each containing 0.05 M NaCl and 0.05% Tween-80; BSA was then added to this solution to a concentration of 1%).

Special reagents

The following were purchased: FMLP, 3,3',5,5'-tetramethyl-benzidine (TMB) substrate tablets (Sigma Chemical Co.), and biotinylated goat anti-human IL-4 (Endogen, Irving, CA). The human rIL-4 and monoclonal anti-human IL-4 Ab that were used for the in-house IL-4 ELISA were kind gifts of Dr. Steven Gillis (Immunex Corporation, Seattle, WA). Polyclonal anti-human IgE Ab was made in a goat and affinity purified. Human rCSa was the kind gift of Dr. Henry Showell (Pfizer, Groton, CT).

Preparation of rHRF

The HrHRF used in these studies was made as previously described (22). Briefly, primers were designed using the sequence of the murine cDNA. These primers were used to generate the human cDNA from the U937 cell line that codes for a protein having a molecular mass of 23 kDa (rp23). The PCR product was expressed in *Escherichia coli* as a protein fused to glutathione-S-transferase, and was affinity purified on immobilized glutathione.

Basophil preparation

Venous blood from consenting donors was anticoagulated with 10 mM of EDTA. Whole blood was centrifuged at 300 × g, and the leukocyte interface (buffy coat) was carefully aspirated and transferred to PAG-EDTA buffer (1:1 v/v). Basophil-enriched suspensions were prepared by Percoll density centrifugation, as previously described (23). Briefly, the diluted buffy coat suspension was layered onto gradients consisting of 12 ml of 55% Percoll layered onto 12 ml of 63% Percoll in clear 50-ml polypropylene centrifuge tubes (Corning, Corning, NY). Percoll gradients were centrifuged at 700 × g for 20 min at room temperature. Basophils were in the fraction consisting of the lower half of the 55% Percoll, 63% interface, and upper half of this layer. Cells in this fraction were washed twice in PAG-EDTA, and had a final wash in cold (4°C) PAG. Basophils were counted in Spiers-Levy chambers using Alcian blue (25). The purity of the basophils in the cell suspensions used for these studies ranged from 5 to 40%. For the experiments using Ag, cells banding at the 55% Percoll interface were removed, washed five times (150 × g for 8 min) in PAG-EDTA to remove platelets and once in cold (4°C) PAG, and used in cultures as basophil-depleted (<1% basophils) cell suspensions.

Culture conditions

Cultures were performed in C-IMDM using 96-well microtiter plates (Costar, Cambridge, MA), as previously described (7). The number of basophils cultured ranged from 50,000 to 250,000 per well. In the experiments using basophil-depleted cell suspensions, the number of total cells added per culture was 500,000. For pretreatment with rHRF, the cells were added to culture wells in 62.5 µl of medium, and brought to 37°C in a CO₂

incubator (5% CO₂) before adding an equal volume of medium (pre-equilibrated in the incubator) containing twice the priming concentration. After 15 min, 125 µl of medium (also pre-equilibrated in the incubator) containing twice the final concentration of stimulus was added to the cell and the cultures. After 4-h incubation, cell-free supernatants were removed for histamine and IL-4 protein analysis by automated fluorimetry and ELISA, respectively (7). To measure IL-13 protein, cell cultures were incubated for 16 to 20 h.

IL-4 and IL-13 ELISAs

Culture supernatants (stored at -80°C) were assayed for IL-4 or IL-13 protein using an in-house IL-4 ELISA and an ultrasensitive (0.8 pg/ml) IL-4 ELISA (Biosource, Camarillo, CA), or by an IL-13 ELISA (Biosource). The commercial kits were performed according to the manufacturer's instructions; the in-house IL-4 method (7) was modified as follows: wells of Immulon-4 microtiter plates (Dynatech Labs., Chantilly, VA) were coated with monoclonal anti-human IL-4 (2.5 µg/ml) in 0.1 M carbonate buffer, pH 9.6, for 48 to 60 h at 4°C. The wells were washed four times with wash buffer (PBS containing 0.05% Tween-20 and 0.01% Thimerosal, pH 7.4). The remaining binding sites were blocked with 150 µl/well of coating buffer containing 1% BSA and a 1/100 dilution of sheep serum for 30 to 60 min at room temperature. Standards (human rIL-4) were made in the C-IMDM used for cell culture. Standards and samples were added to wells in 100 µl. Following a 2-h incubation at 37°C, 5% CO₂, the wells were washed four times with wash buffer. A secondary goat polyclonal anti-human IL-4 Ab conjugated with biotin was diluted in assay buffer (1 µg/ml) and added (100 µl/well) for a 1-h incubation at 37°C, 5% CO₂. Following four washes with wash buffer, streptavidin peroxidase conjugate in assay buffer (1 µg/ml) was added (100 µl/well) and the plates were incubated for 30 min at 37°C, 5% CO₂. After five washes with wash buffer, 100 µl/well of fresh substrate solution was added (10 ml of 0.1 M citrate phosphate buffer, pH 4.3, added to 1 ml of DMSO containing a 1-mg predissolved TMB tablet and 3 µl of 30% H₂O₂). The reactions were developed 10 to 12 min (in the dark) before adding 100 µl/well of stop reagent (2 N H₂SO₄). OD were read using dual wavelengths (450/570 nm). The sensitivity of the in-house ELISA was consistently 4 pg/ml, with a range up to 200 pg/ml.

Results

Dose-dependent enhancement of anti-IgE mediated IL-4 secretion and histamine release by HrHRF

We have shown recently that HrHRF acts as a costimulus of histamine release from basophils not normally releasing to this protein alone (24). As shown in Figure 1, HrHRF also has a significant effect on the secretion of IL-4 protein by these basophils, augmenting the secretion induced by anti-IgE Ab (10 ng/ml) in cells primed with several concentrations of the rHRF. In fact, IL-4 secretion increased by 50 ± 25% when a priming concentration of 80 ng/ml was used, with dose-dependent increases occurring up to 10 µg/ml of the HRF, for an optimal enhancement of 125 ± 20% above control levels (64–475 pg/10⁶ basophils). Histamine released in these cultures was also increased by HRF priming in a dose-dependent fashion from 10 to 70% above controlled release, with a curve that closely resembled that for IL-4 enhancement.

It is important to note that we were concerned initially that the presence of endotoxin contaminating the *E. coli*-derived preparations of rHRF might be responsible for this enhanced secretion occurring in basophils. However, we have been unable to confirm previous reports suggesting that LPS enhances IgE-dependent histamine release (26), nor did we see any correlation between basophil secretion and the amount of endotoxin found in the preparations of HrHRF used in these studies. Furthermore, we have since found that baculovirus-derived HRF, which is essentially free of endotoxin, is also able to enhance IgE-dependent secretion by basophils (data not shown).

Previous studies have shown that IgE-dependent IL-4 protein secretion by basophils is augmented in cells pretreated with IL-3 (7). To rule out the possibility that the HrHRF-priming effect was

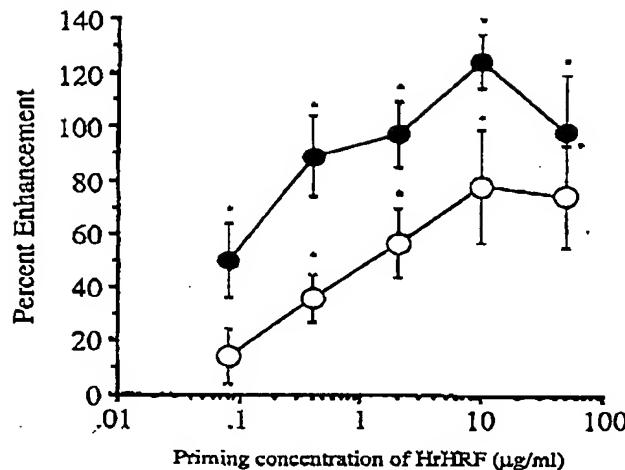


FIGURE 1. Dose-dependent priming with HrHRF enhances IgE-dependent IL-4 protein secretion and histamine release by human basophils. Basophil suspensions were prepared from five donors whose cells do not normally release to HRF. Cell cultures were preincubated with the indicated concentrations of HrHRF for 15 min before challenging with anti-IgE Ab (10 ng/ml). After 4 h in culture, the cellfree supernatants were harvested for histamine (○) and IL-4 protein (●) measurements. Results are reported as the mean \pm SEM of the percentage of enhancement above controlled release using anti-IgE Ab alone. Controlled levels ranged from 64 to 475 pg/10⁶ basophils for IL-4 protein and 19 to 47% of total histamine content. *Indicates statistical significance ($p < 0.05$, $n = 5$) from control values (Wilcoxon test).

due to IL-3 generated by other cell types, experiments were performed with basophils or mixed cell suspensions containing lymphocytes. HrHRF was found not to induce a detectable level of IL-3 protein by either cell type (data not shown).

HrHRF enhances release by basophils activated with a range of anti-IgE Ab concentrations

We next examined whether the priming effect of HrHRF occurs over several concentrations of stimulus or whether it simply causes enhancement of IL-4 by shifting the dose-response curve to anti-IgE. Cells were once again prepared from donors whose basophils did not release to the HRF alone. As shown in Figure 2, these cells were pretreated with the rHRF (10 μ g/ml) and then challenged with 1, 10, or 100 ng/ml of anti-IgE Ab. Basophils from five of the six donors tested showed enhanced IL-4 secretion that was markedly elevated from the levels produced with anti-IgE alone (Fig. 2, a-c). Thus, at 1 ng/ml of anti-IgE Ab, IL-4 secretion among the six donors averaged 25 \pm 11 pg/10⁶ basophils, and this increased 270% for an average of 93 \pm 27 pg/10⁶ basophils following priming with HrHRF. At 10 ng/ml of anti-IgE Ab (which was optimal for IL-4 secretion), there was an overall 64% increase in IL-4 secretion from 137 \pm 47 pg/10⁶ basophils without priming up to 225 \pm 64 pg/10⁶ basophils with HrHRF priming. When 100 ng/ml of anti-IgE Ab was used as stimulus, IL-4 secretion increased 133% from 94 \pm 32 pg/10⁶ basophils (without priming) to 219 \pm 65 pg/10⁶ basophils following priming. For comparison, histamine release in these cultures was similarly enhanced at each concentration of anti-IgE (Fig. 2, d-f).

HrHRF enhances Ag-specific IL-4 secretion by basophils

Since human basophils also secrete IL-4 protein in response to activation with specific Ag (7, 12), we tested whether HrHRF

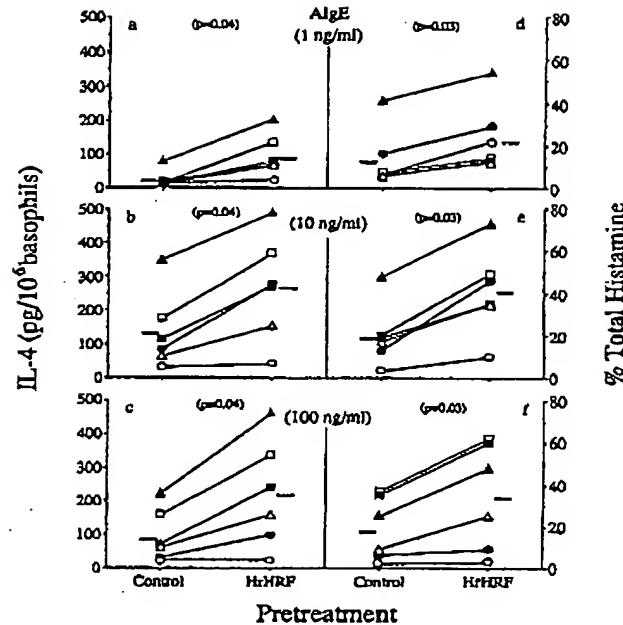


FIGURE 2. Priming of basophils with HrHRF occurs over a wide range of anti-IgE Ab concentrations. Basophil suspensions were prepared from six donors whose cells did not respond to HRF alone. Cell cultures were left untreated (control) or were primed with HrHRF (10 μ g/ml) for 15 min before adding stimulus. After 4 h, the cellfree supernatants were harvested for histamine and IL-4 protein measurements. a-c show the levels of IL-4 protein secreted in response to 1, 10, and 100 ng/ml of anti-IgE Ab, respectively. The corresponding panels d, e, and f show the percentage of total histamine released in these cultures. The IL-4 protein and histamine release data with HrHRF priming were significantly different ($p < 0.05$) from control levels for each concentration of anti-IgE Ab (Wilcoxon test).

priming has a similar effect on this secretagogue. Basophil suspensions were isolated from ragweed allergic donors whose basophils showed no release to the HRF alone. As shown in Figure 3, IL-4 protein was generated in response to two concentrations of Ag (1 and 10 ng/ml) alone, and cells from all five donors secreted enhanced levels of this cytokine following pretreatment with priming concentrations ranging between 3 and 10 μ g/ml of HrHRF (Fig. 3, a and b). Panel c shows that this was similar to that seen with anti-IgE Ab. Although previous studies have demonstrated that activated basophils are the sole source of IL-4 mRNA and protein in mixed leukocyte cultures after 4-h incubation (6, 12), it was possible that the combination of HrHRF and Ag used in these experiments might stimulate the secretion of IL-4 from other cell types. Therefore, basophil-depleted cell suspensions, containing mostly lymphocytes and monocytes, were simultaneously isolated from the same donors and tested for IL-4 secretion in response to HrHRF and Ag stimulation. These mononuclear cell fractions failed to secrete detectable levels of IL-4 after 4 h following the addition of HRF and/or Ag (data not shown).

HrHRF enhances IL-13 secretion from basophils stimulated by anti-IgE Ab

Since human basophils have recently been shown to secrete IL-13 protein in response to cross-linking of the high affinity IgE receptor (8-10), we investigated whether HrHRF would be an additive stimulus for IL-13 production. As shown in Figure 4, HrHRF, at a

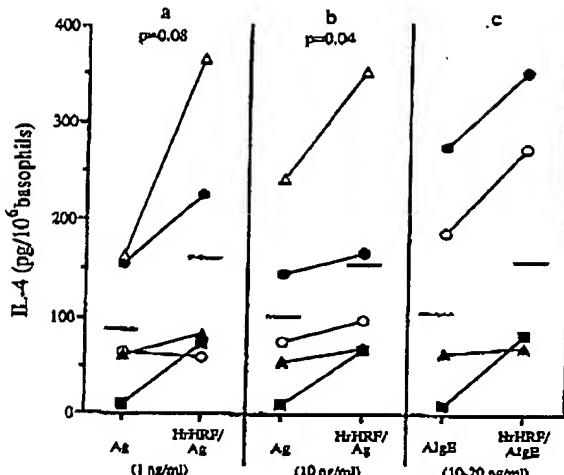


FIGURE 3. Basophil secretion in response to specific Ag is enhanced with HrHRF priming. Basophil suspensions were prepared from five donors whose cells released histamine in response to ragweed Ag, but not to HrHRF. Cell cultures were left untreated or were primed with HrHRF (3-10 μ g/ml) for 15 min before adding specific Ag at 1 and 10 ng/ml final concentration (a and b, respectively). After 4 h, the cell-free supernatants were harvested for IL-4 protein measurements. For comparison, basophils from these same donors (except the donor represented by the opened triangle) were challenged with anti-IgE Ab (10 ng/ml) following pretreatment with and without HrHRF (c). The IL-4 protein data with HrHRF priming were significantly different ($p < 0.05$) from control levels for cells challenged with 10 ng/ml of Ag (Wilcoxon test).

dose of 10 μ g/ml, enhanced IL-13 production for an average of 40% from 226 ± 110 pg/10⁶ basophils to 316 ± 117 pg/10⁶ basophils in five of five donor basophils stimulated with 10 to 20 ng/ml of anti-IgE Ab. HrHRF used alone did not cause any detectable IL-13 secretion by these donor cells.

The effect of HrHRF priming on basophils activated by IgE-independent stimuli

We have reported previously that the basophil secretagogues C5a or FMLP peptide induce little to no IL-4 secretion from basophils after 4-h stimulation (7). Therefore, in a final series of experiments, we examined whether priming with the HRF might affect the ability of these IgE-independent stimuli to acquire the ability to induce the secretion of IL-4 protein. We were unable to detect IL-4 protein following activation with three different concentrations of C5a, which confirmed our previous findings. Cells from several donors, however, did secrete detectable levels of cytokine after pretreatment with HrHRF, with cultures costimulated with one dose (20 ng/ml) of C5a, showing a slight increase compared with control cultures receiving C5a alone (data not shown). It is important to note, however, that the levels of IL-4 protein were barely above detection and were nearly 10-fold less than that achieved with IgE-dependent activation.

Others have shown that basophils secrete higher levels of IL-4, although at a slower rate, if the cells are cultured for 24 h in media containing both C5a and IL-3 (27). Since HrHRF may affect basophil function similarly to that seen with IL-3 priming, we also cultured basophils with HrHRF and C5a for longer time periods (up to 24 h) to test whether greater levels of IL-4 are generated with this combination. However, cells cultured 24 h with HrHRF

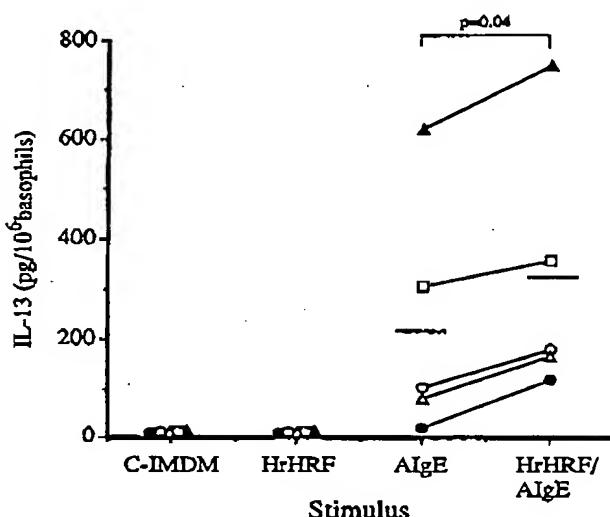


FIGURE 4. IgE-dependent IL-13 protein secretion by basophils is enhanced with HrHRF priming. Basophil suspensions were prepared from five donors whose cells did not react to HrHRF alone. No IL-13 was detected when cells were incubated with medium alone (C-IMDM) or with HrHRF alone. Cell cultures were left untreated (AlgE) or were primed with HrHRF (HrHRF/AlgE) for 15 min before adding stimulus for an additional 16 to 20 h. Cell-free supernatants were then harvested for IL-13 protein measurements. The IL-13 protein data with HrHRF priming were significantly different from that produced in control cultures receiving anti-IgE Ab (10-20 ng/ml) alone (Wilcoxon test).

and C5a did not generate IL-4 protein to any greater extent than that secreted after 4 h of culture (data not shown).

The effect of HrHRF on cells stimulated with FMLP peptide is shown in Figure 5. This potent basophil secretagogue induced little to no IL-4 protein secretion when used alone, and at 10^{-8} , 10^{-7} , or 10^{-6} M concentrations, and priming with HrHRF caused no apparent enhancement in the levels of IL-4 induced by this stimulus (a, b, and c). Likewise, there were no consistent changes in the amount of histamine released by these cultures (d, e, and f).

Discussion

For the past decade, much of our work has focused on the characterization of an HRF that causes mediator release from basophils isolated from selected atopic donors in a reaction requiring the expression of a particular type of IgE that we refer to as IgE⁺. The clinical relevance of this HRF in the pathogenesis of allergic disease is indicated by the fact that a similar histamine-releasing activity is found in lung, nasal, and skin lavage fluids, particularly those taken during late phase reactions to Ag challenge (21, 28, 29). Furthermore, PBMCs isolated from children having severe atopic dermatitis and food sensitivity generate an IgE-dependent HRF and have basophils that spontaneously secrete histamine. The symptoms, release of histamine, and the generation of HRF in these individuals were diminished with removal of the food responsible for these activities (30).

Recent studies show that, in the presence of IgE⁺, HrHRF acts as a complete stimulus, causing the release of histamine and IL-4 protein from basophils isolated from selected atopic donors (23). Although the exact nature for the interaction between so-called IgE⁺ and HRF remains unknown at this time, the present study strongly suggests that the latter is capable of affecting the function

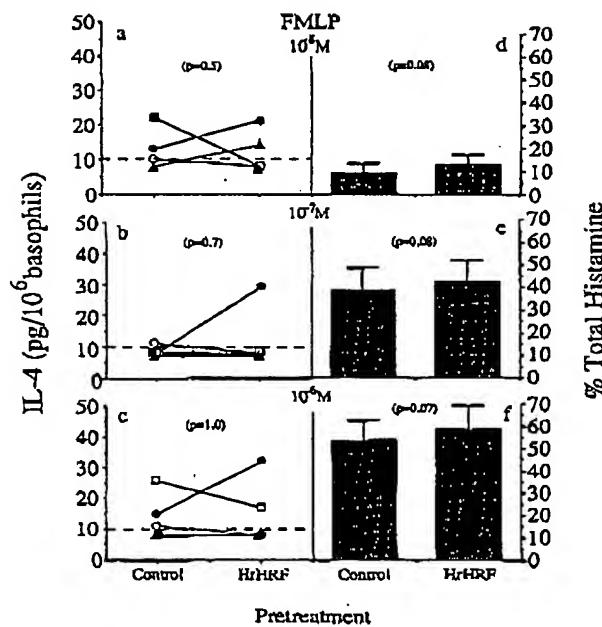


FIGURE 5. Priming with HrHRF does not induce IL-4 secretion by basophils challenged with FMLP peptide. Basophil suspensions were left untreated (control) or were primed with HrHRF for 15 min before adding stimulus. After 4 h, the cell-free supernatants were harvested for histamine and IL-4 protein measurements. a–c show the levels of IL-4 protein secreted in response to 10^{-6} , 10^{-7} , and 10^{-8} M concentrations of FMLP peptide, respectively. The corresponding panels d, e, and f show the mean \pm SEM ($n = 5$) percentage of total histamine released in these cultures. IL-4 protein and histamine release levels obtained with HrHRF priming were not significantly different ($p > 0.05$) from control cultures (Wilcoxon test).

of all basophils. Thus, in the presence of IgE⁺, HrHRF acts by modulating the response of basophils to subsequent activation with a costimulus, such as anti-IgE Ab or specific Ag. These findings, in fact, suggest that HRF may interact with a specific receptor, other than IgE⁺. With respect to this belief, HRF may function more like a cytokine by binding a specific receptor that is found on basophils and most likely other cells that participate in allergic inflammation. It is important to note that in the presence of IgE⁺, HrHRF and anti-IgE Ab (or Ag) show an additive effect on basophil secretion, such that the amount of IL-4 or histamine generated in response to this combination is more equal to the sum of the two alone (24). In this instance, the expression of IgE⁺ itself seems to change the activation state of the cell, such that a costimulus is not necessary for secretion.

In investigating the effects of HrHRF priming on the release of histamine from basophils stimulated by a variety of secretagogues, IgE-dependent stimulation was most affected compared with the effects on activation by IgE-independent mediator release (24). In this study, we confirmed the finding that IgE-mediated histamine release is enhanced with HrHRF priming and, in addition, found that IL-4 and IL-13 secretion are similarly affected. Interestingly, HrHRF priming was found to have no significant effect on histamine release or cytokine secretion when cells were stimulated by the IgE-independent stimuli, such as the FMLP peptide. The observation that HrHRF is more effective in modifying IgE-dependent rather than IgE-independent stimulation is not fully understood, but may possibly result from an ability of this HRF to

generate intracellular signals that are linked closely to those mediated through the high affinity receptor (Fc ϵ RI). In theory, such a receptor for HRF may function as a result of close proximity with Fc ϵ RI on the surface of basophils, much like CD3 is to the TCR. Whether this receptor hypothesis or an alternative theory is correct for the mechanism of action by HRF, the fact that this protein is capable of modifying the function of all basophils (and most likely other cell types) would suggest that it has a significant role in the pathogenesis of allergic diseases.

Acknowledgments

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IMMUNOBIOLOGY

Human recombinant histamine-releasing factor activates human eosinophils and the eosinophilic cell line, AML14-3D10

Roy Bheekha-Escura, Donald W. MacGlashan Jr, Jacqueline M. Langdon, and Susan M. MacDonald

The human recombinant histamine-releasing factor (HrHRF) was previously shown to induce histamine release from human basophils from a subset of donors. The ability of HrHRF to directly induce histamine release from only certain basophils was thought to involve interaction between HrHRF and a particular kind of IgE, termed IgE⁺, on the surface of these cells. Recent studies disproved the hypothesis that the IgE molecule or its high-affinity receptor, Fc ϵ RI, is involved in secretion of histamine and cytokines by basophils stimulated with HrHRF. Rather, data suggest that HrHRF is a cytokine that stimu-

lates basophils by binding to a cell-surface structure other than the IgE molecule. This report describes the effects of HrHRF on another inflammatory cell type: eosinophils from mildly allergic donors. In purified eosinophils primed with granulocyte-macrophage colony-stimulating factor, both tumor necrosis factor α (TNF- α) and HrHRF induced increased secretion of interleukin (IL)-8. In addition, both HrHRF and IL-5 enhanced secretion of IL-8 stimulated by TNF- α . Secretion of IL-8 reached a plateau level in less than 24 hours, was inhibited by cycloheximide, and required the pres-

ence of HrHRF throughout the culture period. In some eosinophil preparations, HrHRF induced calcium mobilization that was inhibited by pertussis toxin. Additionally, HrHRF caused secretion of IL-8 from the human eosinophilic cell line, AML14-3D10, which does not possess the α chain of Fc ϵ RI. These data provide evidence that HrHRF contributes to activation of eosinophils and thus suggest an additional role for HrHRF in the pathophysiological mechanisms of allergic disease. (Blood. 2000;96:2191-2198)

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Introduction

Histamine-releasing factors (HRFs) comprise a group of molecules that induce basophil degranulation. Molecules with this activity include interleukins,^{1,2} chemokines,³⁻⁵ and the previously subcloned IgE-dependent HRF.⁶ This human recombinant HRF (HrHRF) (also known as p23⁷ or translationally controlled tumor protein^{8,9}) was initially described as a complete secretagogue for secretion of histamine⁶ and interleukin (IL)-4¹⁰ from basophils from a subset of allergic donors. Mediator release was thought to be the consequence of physical interaction between HRF and a certain type of IgE, termed IgE⁺, on the surface of the responding basophils.¹¹ However, several lines of evidence suggest that the interaction with IgE may not be required for cell activation mediated by HrHRF. First, HrHRF enhanced anti-IgE-mediated histamine release, as well as IL-4 and IL-13 protein production, in donors with IgE⁻ on the surface of their basophils, which by definition do not respond directly to HrHRF.¹² Second, in IgE⁺ donors, HrHRF-induced histamine release can be modulated by agents that do not affect IgE-dependent histamine release.¹³ Third, rat basophilic leukemia cells transfected with the human α , β , and γ chains of Fc ϵ RI released histamine to polyclonal anti-IgE after passive sensitization with IgE^{*}, but no histamine release occurred when HrHRF was the stimulus.¹⁴ These data indicate that HrHRF exerts its activity independently of IgE and strongly suggest the existence of an HrHRF-specific activation pathway other than through Fc ϵ RI. Therefore, we studied the role of HrHRF in activation of human

eosinophils, inflammatory cells that do not normally express cell-surface Fc ϵ RI.

Although one study found that eosinophils from a subset of hypereosinophilic patients have Fc ϵ RI¹⁵ and another showed that the Fc ϵ RI α chain is up-regulated on eosinophils after antigen challenge in asthmatic subjects,¹⁶ this area of investigation remains controversial. A more recent study demonstrated that eosinophils from a variety of donors, including mildly allergic subjects, possess intracellular Fc ϵ RI α chain, but it was undetectable on the cell surface.¹⁷ Additionally, although Kita et al¹⁸ found extremely low levels of surface Fc ϵ RI, there was no effector function that could be attributed to this receptor. For these experiments, we used eosinophils from mildly allergic patients who had no detectable surface expression of Fc ϵ RI, the low-affinity IgE receptor Fc ϵ RII, or IgE, as determined by flow cytometry. Furthermore, we used the human eosinophilic cell line AML14-3D10, which has no observable Fc ϵ RI α chain on Western blot analysis of cell lysates.

Eosinophils are known to contribute to the pathophysiological mechanisms of allergic diseases by secreting proinflammatory granule proteins, such as major basic protein and eosinophil cationic protein, that induce damage of bronchial epithelial cells.^{19,20} Increased numbers of these cells are found in bronchoalveolar lavage (BAL) fluids and bronchial biopsy specimens from allergic asthmatic patients during the late phase of an allergic reaction (LPR).²¹ There is evidence that these cells are capable of synthesizing, storing, and in some cases, releasing cytokines that contribute

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to cell recruitment and activation. Transcription or translation of the following cytokines has been reported to occur in eosinophils: IL-1 α , IL-3, IL-5, IL-6, IL-8, transforming growth factor (TGF) α and TGF- β , macrophage inflammatory protein 1 α (MIP-1 α), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF- α),²² IL-4,²³ IL-10,²³ IL-16,²⁴ the regulated upon activation, normal T-cell expressed and secreted (RANTES) cytokine,²⁴ and IL-12.²⁵ Although there is controversy about which of these cytokines are actually secreted,²⁶ the eosinophils, but not the neutrophils, of patients with bronchial asthma or atopic dermatitis show up-regulated IL-8 protein.²⁷ Furthermore, concentrations of IL-8 in BAL fluids from asthmatic patients are several times higher than in those from healthy subjects.²⁸ Although IL-8 was originally defined as a neutrophil chemoattractant in vivo,^{29,30} it was found that eosinophils show chemotactic responses toward IL-8 in vitro³¹ and that this function is enhanced in eosinophils from antigen-challenged allergic subjects.³² Taken together, these observations suggest that the increased levels of IL-8 in BAL fluids from asthmatic patients may be at least partly due to eosinophil activation that could lead to further eosinophil recruitment to the inflammatory site.

In this study, we demonstrated that HrHRF directly caused the release of IL-8 or enhanced TNF- α -induced IL-8 secretion from human eosinophils and the eosinophilic AML14-3D10 cell line, primed with GM-CSF. Enhancement of cytokine secretion depended on both protein synthesis and the presence of HrHRF throughout the culture period. Additionally, HrHRF was chemotactic for eosinophils. In eosinophils from some donors, HrHRF also induced calcium (Ca^{++}) mobilization, which was independent of the addition of exogenous GM-CSF or TNF- α .

Materials and methods

We used 10 \times piperazine diethanesulfonic acid (PIPES) buffer that contained 250 mmol/L PIPES (Sigma, St Louis, MO), 110 mmol/L sodium chloride (NaCl), and 50 mmol/L potassium chloride (KCl), adjusted to pH 7.4. PIPES-albumin-glucose (PAG) buffer was made with 10% 10 \times PIPES and contained 0.003% human serum albumin (Calbiochem-Novex Biochem Corp, La Jolla, CA) and 0.1% D-glucose. PAG-calcium-magnesium (PAGCM) buffer was made by adding 1 mmol/L calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) to PAG buffer. Percoll (Pharmacia, Piscataway, NJ) was made isotonic by mixing 1 part 10 \times PIPES with 9 parts Percoll. This solution was then adjusted to a density of 1.09 g/mL by adding approximately 38 mL of 1 \times PIPES to 100 mL of isotonic Percoll and verifying the results with a densitometer.³³

For preparation of cell lysates, 1 mL of lysis buffer was used; this consisted of 25 mmol/L HEPES, 5 mmol/L KCl, 119.4 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L $CaCl_2$ (pH 8), 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, and 20 μ L protease inhibitor mixture (PharMingen, San Diego, CA). GM-CSF, TNF- α , IL-5, and RANTES were purchased from R&D Systems (Minneapolis, MN).

HrHRF production

HrHRF was subcloned from the PGEX-2T vector by using the restriction enzymes *Eco*R1 and *Bam*H1 used in *Escherichia coli* production.⁶ The 524 base-pair (bp) fragment was ligated into the baculovirus vector pBlueBac III, which coexpresses β -galactosidase, for color selection of successful recombination. In accordance with the manufacturer's specifications for the baculovirus system (MAXBAC; Invitrogen, San Diego, CA), plasmid DNA was transfected into SF9 cells for viral isolation and amplification and High 5 insect cells were subsequently used for protein production. The insect cells were grown commercially on a large scale in serum-free medium (Paragon Biotech, Baltimore, MD). The cell pellet from 14 L of insect cells

was dissolved in 400 mL of NBB buffer (20 mmol/L sodium phosphate and 500 mmol/L NaCl [pH 7.8]) to which 400 μ L of inhibitor mixture (PharMingen) was added. The suspension was freeze-thawed twice and centrifuged at 9000 rpm for 20 minutes. Purification of the supernatant, which contained the HrHRF protein, was accomplished by 2-step column chromatography. First, the supernatant was mixed with Dc-52 (Whatman, Maidstone, UK) in 0.02 mol/L Tris buffer (pH 8). The flow-through was screened for the presence of HrHRF by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using a polyclonal anti-HRF antibody generated against the recombinant material produced in *E. coli*.⁶ Positive fractions were concentrated and placed on a column (Sephadex G75; Pharmacia) in physiologic PIPES buffer. Again, SDS-PAGE and Western blotting were used to screen the column fractions. Fractions were pooled and concentrated and the bioactivity was confirmed by using the basophil histamine-release assay. The protein concentration of HrHRF was determined with a protein assay (Bio-Rad, Hercules, CA) and was found to be 160 μ g/mL. Because the molecular weight of HrHRF is 23 kd, this stock solution is equivalent to 7 μ mol/L. HrHRF was dialyzed against physiologic PIPES buffer for use in all assays. HrHRF had a single band on SDS-PAGE and was judged to be more than 95% pure.

Endotoxin levels in purified HrHRF were determined by a limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD), according to the manufacturer's specifications. Additionally, endotoxin was removed from certain aliquots of HrHRF by using a column containing polymyxin B immobilized on agarose (Detoxi-Gel; Pierce, Rockford, IL).

Purification of eosinophils

Granulocytes were isolated from EDTA-anticoagulated venous blood from mildly allergic donors by gradient centrifugation in isotonic Percoll (1.09 g/mL). Red blood cells were removed by hypotonic lysis, and CD16-positive cells (neutrophils) were removed with an immunomagnetic bead technique.³³ Eosinophils were differentiated by using high-power light microscopy after staining (Diff-Quick Stain Kit; Dade, Düsseldorf, Switzerland). Eosinophil purity was always greater than 98%; in some experiments, it was 100%.

Cell cultures

Human eosinophils. Purified eosinophils were resuspended in medium M199 (Gibco, Grand Island, NY) containing 20% fetal-calf serum (FCS; Sigma) with or without GM-CSF (10 ng/mL) and incubated for 30 minutes at 37°C in 5% carbon dioxide (CO₂). Aliquots of 5 \times 10⁵ cells were transferred to 48-well plates containing TNF- α (50 ng/mL or 10 ng/mL), IL-5 (50 ng/mL), HrHRF (0.7 μ mol/L), or a combination of these reagents. The final eosinophil concentration in each assay was 2 \times 10⁶/mL. In some experiments, either pertussis toxin (PT) or its inactive β -oligomer (List Biological Laboratories, Campbell, CA) was also added (1 ng/mL each). After 24 hours in culture at 37°C in 5% CO₂, plates were centrifuged for 5 minutes at 1000 rpm and supernatants were collected and stored at -20°C until analysis by enzyme-linked immunosorbent assay (ELISA). In some experiments, cycloheximide (1 μ mol/L) (Sigma) was added at the beginning of the culture. Cycloheximide did not affect cell viability as determined by staining with erythrosin B (Sigma).

AML14-3D10 cells. The AML14-3D10 subline, a gift of Dr Cassandra Paul (Wright State University, Dayton, OH), is a human eosinophilic leukemic cell line that consistently shows eosinophilic granules in 95% of its cells.³⁴ Cells were cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD), 8% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μ g/mL gentamicin (all from BioWhittaker), and 5 \times 10⁻⁵ mol/L mercaptoethanol (JT Baker, Phillipsburg, NJ). Cells were incubated with the same cytokines used with the human eosinophils and cultured in the same manner. The final concentration of cells in the IL-8 production assays was 2 \times 10⁶/mL.

IL-8 determination

Supernatants from stimulated eosinophils were assayed with an IL-8-specific ELISA (Biosource, Camarillo, CA), according to the manufacturer's specifications. The threshold for cytokine detection was 10 pg/mL. In some cases, the values obtained were compared with values determined by using another commercial kit (R&D Systems). No significant differences were observed between the 2 assays.

Chemotaxis experiments

Chemotaxis experiments were performed by using the modified Boyden chamber technique described previously.³⁴ Briefly, 25 μ L of PAGCM buffer or various concentrations of the stimuli in the same buffer were placed in the lower chamber in triplicate. A 5- μ m pore-sized polycarbonate membrane (Nuclepore Corp, Pleasanton, CA) separated the upper and lower chamber. Eosinophils (10^6) resuspended in PAGCM buffer were placed in each well of the upper chamber on top of the membrane. The chamber was then incubated for 30 minutes at 37°C in 5% CO₂ and air, after which the chamber was disassembled. The membrane was removed, washed in PAG buffer to remove the nonmigrating eosinophils from the upper surface, scraped, and stained with Wright stain. Eosinophils from 10 high-power fields of triplicate wells were identified and counted.

Ca⁺⁺ mobilization assay

The Ca⁺⁺ mobilization assay we used was a modification of a procedure described for human basophils by MacGlashan et al.³⁶ Purified eosinophils were loaded with 1 μ mol/L Fura 2-AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C in RPMI-1640 (Gibco BRL) containing 2% FCS and 0.32 mmol/L EDTA. In some experiments, eosinophils were incubated for 2 hours at 37°C in 5% CO₂ with 1 ng/mL of PT or its β -oligomer and Fura 2-AM was added during the last 30 minutes of the incubation. The cells (5×10^5) were washed once with PAG buffer and resuspended in 200 μ L of PAG for loading in the microscope observation chamber. A 15- μ L cell suspension was placed on a siliconized (Sigma Cote; Sigma) coverslip that comprised the base of the observation chamber. After 5 to 10 minutes of settling time, the cell drop was overlaid with 1 mL of PAGCM buffer at 37°C. The temperature, which was measured by a probe placed next to the settled cells, was brought to a stable 36.5°C and the stimulus (dissolved in 1 mL of prewarmed buffer) was added. Intracellular changes in Ca⁺⁺ were monitored with a Zeiss Axiovert microscope with epifluorescence capacity as described previously.³⁷

Flow cytometry to determine surface IgE expression

Cells were incubated for 30 minutes at 4°C in phosphate-buffered saline containing 0.2% bovine serum albumin (PBS-BSA) and 3.6 mg/mL human IgG with saturating concentrations of receptor-specific antibody or an equivalent concentration of an irrelevant isotype-matched control antibody. Surface IgE was detected by using a fluorescein isothiocyanate-conjugated polyclonal goat antihuman IgE (Kirkegaard and Perry, Gaithersburg, MD). The Fc ϵ RI α chain was investigated by using an IgG₁ mouse antihuman monoclonal antibody (mAb), 22E7 (provided by Dr J. Kochan, Hoffman-La Roche Inc, Nutley, NJ). Fc ϵ RII (CD23) was assessed by using mAb 9P.25 (Immunotech Inc, Westbrook, ME). Anti-Fc ϵ RI mAb 22E7 is known to detect the high-affinity IgE receptor on human basophils,³⁸ and anti-Fc ϵ RII mAb 9P.25 detects the low-affinity IgE receptor on human B cells.³⁹ An IgG₁ isotype control mAb was purchased from Sigma. Cells were washed with PBS-BSA, incubated with 1:150 dilutions of R-phycocerythrin-conjugated F(ab')₂ goat antimouse IgG antibody (Biosource) for 30 minutes at 4°C in the dark, washed, resuspended in PBS with 0.2% BSA, and assayed immediately with a flow cytometer (EPICS Profile II; Coulter, Hialeah, FL). Results were expressed as mean fluorescence intensity.

Cell lysates and Western blots to determine the presence of the Fc ϵ RI α chain

Human basophils were isolated by negative selection using magnetic beads from a basophil isolation kit (Miltenyi, Auburn, CA).¹³ Basophils (5.5×10^6)

lysing in purity from 67% to 97% or AML14-3D10 cells (5.5×10^6) were lysed in 220 μ L of lysis buffer for 20 minutes on ice. Lysed cells were spun at 14 000 rpm for 15 minutes in an Eppendorf centrifuge (Brinkman, Westbury, NY). Subsequently, 20 μ L of each cell type (approximately 500 000 cells/lane) were combined with 20 μ L of Tris-glycine 2 \times sample buffer (Novex, San Diego, CA), boiled for 5 minutes, and electrophoresed (125 V) for 2 hours on a 4% to 20% Tris-glycine gel (Novex). SeeBlue molecular-weight markers (Novex) were used as a standard. Proteins from the cell lysates were transferred on nitrocellulose (Schleicher & Schuell, Keene, NH; 30 V for 2 hours). The nitrocellulose was blocked in 10% nonfat dry milk in PBS with 0.5% Tween (PBS-T) for 1 hour. The blot was washed 3 times in PBS-T and incubated in PBS-T and 2% BSA for 3 hours with mAb 22E7 ascites, which detects the Fc ϵ RI α chain, at a dilution of 1:300. After a 15-minute washing, the secondary antibody, sheep antimouse horseradish peroxidase (Amersham Life Sciences, Piscataway, NJ), was diluted 1:20 000 in PBS-T and incubated for 1 hour. After the final washing step (6 times for 6 minutes each in PBS-T), the blot was developed by using SuperSignal chemiluminescent substrate (Pierce) and exposed to Hyperfilm ECL (Amersham Life Sciences).

Statistical methods

Results are expressed as the mean \pm SEM. Where indicated, cells incubated under different conditions were compared by using the Student *t* test. In the Ca⁺⁺ mobilization experiments, the Student *t* test was used to compare time points before and after the addition of the stimulus. Specifically, the *t* test was used to compare the area under the curve for a relevant time period by using the initial phase of the calcium response, which is important in the PT response.

Results

IL-8 production by HrHRF in eosinophils requires priming with GM-CSF

To determine the optimal conditions for cytokine production by eosinophils stimulated with HrHRF, freshly isolated eosinophils were incubated with HrHRF, TNF- α , and IL-5 alone and with a combination of these 3 cytokines. None of these cytokines alone induced significant IL-8 secretion. Only the combination of TNF- α and IL-5 induced significant cytokine production, which was only 400 pg/mL greater than the value for the medium control (data not shown). Although significant IL-8 production by peripheral blood eosinophils stimulated with TNF- α alone has been reported,²³ other authors showed that priming of eosinophils with GM-CSF is required for optimal IL-8 secretion.²⁴ Therefore, we tested whether preincubation of eosinophils with GM-CSF would alter IL-8 secretion. Incubation of eosinophils from 4 separate donors with GM-CSF for 30 minutes before stimulation greatly enhanced the responsiveness of these cells to TNF- α but not to IL-5 (Figure 1). In the presence of GM-CSF, HrHRF induced some IL-8 production above the value for the medium control; however, HrHRF enhanced IL-8 secretion induced by TNF- α or IL-5. The magnitude of the enhancement with HrHRF and TNF- α was greater than that with HrHRF and IL-5. Additionally, this enhancement of IL-8 production was comparable to that induced by the combination of TNF- α and IL-5 (Figure 1).

Although the HrHRF preparations produced in baculovirus used for these experiments contained low but variable amounts of endotoxin, we do not believe that contaminating endotoxin accounted for our results. Although there is one report that 10 ng/mL of lipopolysaccharide (LPS) induced IL-8 secretion from human eosinophils,⁴⁰ we used a polymyxin B column to remove endotoxin and our HrHRF preparation contained 10 000-fold less endotoxin

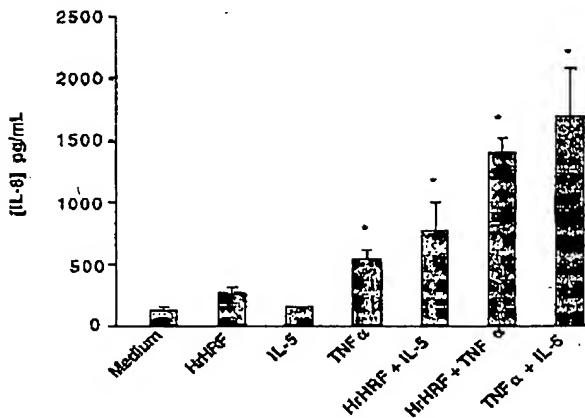


Figure 1. Augmentation of interleukin (IL) 8 production in eosinophils primed with granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were preincubated in 10 ng/mL GM-CSF. After 30 minutes, aliquots were distributed in wells containing medium, 50 ng/mL tumor necrosis factor α (TNF- α), 50 ng/mL IL-5, 0.7 μ mol/L human recombinant histamine-releasing factor (HrHRF), or a combination of these cytokines, as indicated on the x-axis. After 24 hours of culture, supernatants were collected and IL-8 content was determined by enzyme-linked immunosorbent assay (ELISA). * $P < .05$ compared with cells incubated with medium alone (4 experiments).

than the amount reported as necessary to induce IL-8. Thus, HrHRF that contained 8.5 ng/mL of endotoxin on limulus amoebocyte lysate assay was placed on a Detoxi-Gel column containing polymyxin B immobilized on agarose and an 8500-fold reduction in endotoxin was achieved. This resulted in a barely detectable level of 1 pg/mL of endotoxin in the assay with eosinophils. Additionally, preparations that contained almost undetectable amounts of endotoxin (1 pg/mL) behaved similarly to those with higher levels of endotoxin (350 ng/mL). Moreover, when preparations of HrHRF containing 350 ng/mL of endotoxin were used, LPS at a concentration of 350 ng/mL did not reproduce the results ($n = 4$, data not shown).

Although HrHRF alone did not significantly increase IL-8 production in the 4 donor samples used to produce the results shown in Figure 1, there was a trend toward an increase. Therefore, we examined samples from additional donors. In 12 experiments using eosinophil samples from 7 different donors, HrHRF induced significant production of IL-8 by eosinophils primed with GM-CSF, even in the absence of TNF- α (Figure 2). All 12 experiments are plotted in Figure 2, and the same symbol is used to depict samples from the same donor. Although there was some variability, mean IL-8 levels were 250 ± 136 pg/mL without stimulation and 682 ± 202 pg/mL after exposure to HrHRF; thus, HrHRF significantly increased production of IL-8 ($P < .03$). Because of these results, priming or coculture with GM-CSF was used in subsequent experiments that demonstrated IL-8 production from eosinophils, the Ca^{++} experiments, and the studies with AML14-3D10 cells. Additionally, the kinetics of cytokine production was studied by collecting supernatants of stimulated cells at 5, 16, and 24 hours. As shown in Figure 3, the kinetics of IL-8 secretion was similar regardless of the stimulus used and reached a plateau level in less than 24 hours.

IL-8 production by eosinophils requires protein synthesis

It is known that some cytokines are both stored in a preformed state in granules and newly synthesized. For example, TNF- α is released from mast cells after cross-linking Fc ϵ RI as a result of both release

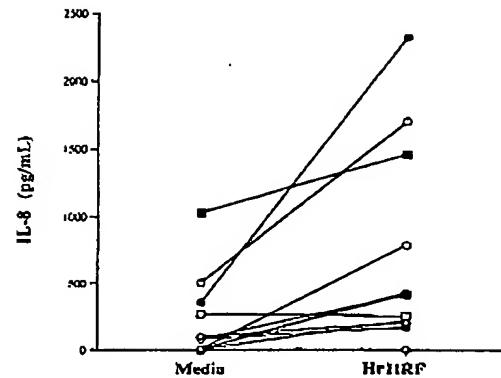


Figure 2. Induction of IL-8 secretion by HrHRF alone in eosinophils primed with GM-CSF. Eosinophils were treated with GM-CSF and placed in medium alone or with 0.7 μ mol/L HrHRF, and the supernatants were tested for IL-8 content. Twelve experiments using samples from 7 donors are represented. Each experiment is plotted; the same symbol is used to depict samples from the same donor. There was a significant difference in IL-8 production between HrHRF-stimulated cells and controls ($P < .03$).

from preformed cytoplasmic granule stores and de novo synthesis.⁴¹ We questioned whether de novo protein synthesis is required for cytokine-induced IL-8 production from eosinophils. Stimulated eosinophils were incubated in the presence or absence of 1 μ mol/L cycloheximide. As shown in Figure 4, cycloheximide inhibited IL-8 production by all the stimuli. However, in 2 of 3 experiments, the combination of HrHRF and TNF- α induced small but measurable amounts of IL-8 from cycloheximide-treated cells. Because cycloheximide did not decrease cell viability, the decrease in HrHRF-stimulated IL-8 production was probably due to decreased protein synthesis.

Continued presence of HrHRF in cultures is necessary for enhanced IL-8 production

We questioned whether incubation of eosinophils with HrHRF for short periods would provide the necessary signal to enhance IL-8 secretion induced by TNF- α . Therefore, eosinophils treated with GM-CSF were incubated for 1 hour with medium or HrHRF, washed, and stimulated with additional medium or TNF- α without HrHRF. The culture continued for 24 hours. When HrHRF was

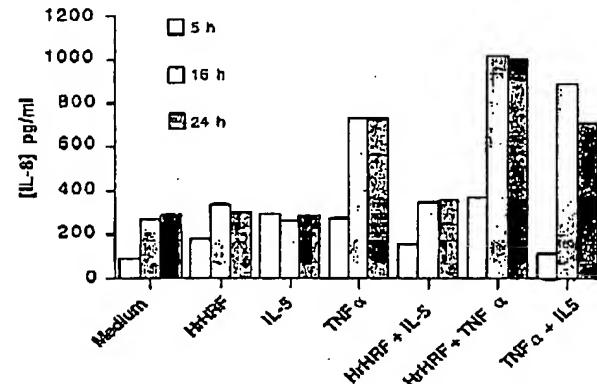


Figure 3. Kinetics of IL-8 production. Eosinophils were incubated with GM-CSF for 30 minutes and then stimulated for 5 hours (open bars), 16 hours (gray bars), or 24 hours (closed bars) with the cytokines indicated on the x-axis. An experiment representative of 3 is shown.

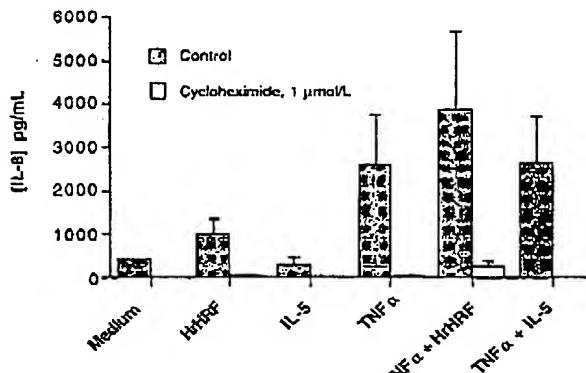


Figure 4. IL-8 production is dependent on protein synthesis. Eosinophils were incubated in the presence of GM-CSF and cycloheximide (1 μ mol/L; open bars) or GM-CSF alone (closed bars). After 30 minutes, cells were transferred to wells containing the stimuli indicated on the x-axis. Twenty-four hours later, the IL-8 content of the supernatants was determined by ELISA (3 experiments).

present during only the first hour of culture, no enhancing effect on TNF- α -induced IL-8 production was observed (1963 pg/mL versus 1778 pg/mL). In contrast, when HrHRF was present throughout the culture period, 3913 pg/mL of IL-8 was generated. Decreased IL-8 production in cells primed with HrHRF was not a consequence of cell washing because comparable levels of IL-8 were produced in cultures that had been washed and then replenished with the appropriate cytokines (3848 pg/mL versus 3913 pg/mL).

IL-8 production by stimulated eosinophils is inhibited by a modulator of G_{α_i} protein activity

We previously found evidence that HrHRF-induced histamine release from human basophils is insensitive to PT, a known inhibitor of G proteins bearing an α_i subunit.¹³ In contrast to these results with basophils, PT (1 ng/mL) moderately reduced IL-8 secretion from GM-CSF-primed eosinophils that was induced by HrHRF, TNF- α , or the combination of HrHRF and TNF- α (Figure 5). This reduction was a consistent finding. To provide a control, cells were also stimulated in the presence of the PT β -oligomer that does not affect G_{α_i} function. In the experiments shown in Figure 5, the PT β -oligomer did not affect IL-8 secretion induced by the various stimuli. These experiments used a suboptimal concentration of TNF- α (10 ng/mL), which was also shown to have synergy with HrHRF for IL-8 production.

HrHRF induces Ca^{++} mobilization in eosinophils from some donors that is inhibited by PT

Because human eosinophils are known to possess the receptor for GM-CSF,⁴² we investigated the effect of HrHRF on the Ca^{++} response in the absence of GM-CSF. Unlike the previous experiments, the initial Ca^{++} experiments did not include use of GM-CSF to prime the eosinophils. Figure 6 shows the average Ca^{++} response of eosinophils (4 different donors) preincubated with HrHRF in the presence of PT or the PT β -oligomer. HrHRF induced an elevation in Ca^{++} in eosinophils preincubated with the PT β -oligomer ($P < .05$), whereas in cells pretreated with PT, the Ca^{++} response induced by either HrHRF or platelet-activating factor (PAF), the positive control, was completely ablated. Under these experimental conditions, Ca^{++} mobilization occurred in 4 of 6 samples from different donors tested. However, in samples from a

total of 9 donors examined over months, Ca^{++} responses to HrHRF occurred in only 25% (9 of 36 experiments). The lack of responsiveness of some cell preparations was not due to a general inability of the cells to mobilize Ca^{++} because eosinophils always responded to stimulation with PAF, regardless of the effect of HrHRF. In general, the HrHRF-induced Ca^{++} response was less vigorous than that induced by PAF.

Because HrHRF induced a Ca^{++} response only 25% of the time, we subsequently preincubated the cells with GM-CSF. Preincubation of cells with GM-CSF, TNF- α , or both did not change the response to HrHRF, nor did it induce Ca^{++} mobilization in cells otherwise unresponsive to HrHRF (data not shown). Again, LPS used at concentrations of up to 500 ng/mL as a control for endotoxin in the preparation failed to induce Ca^{++} mobilization in eosinophils (data not shown).

HrHRF is chemotactic for human eosinophils in vitro

Teshima et al⁴³ reported that recombinant p26 HRF injected into the peritoneum of ovalbumin-sensitized mice caused eosinophil recruitment within 4 hours. Because of this report, we performed eosinophil chemotaxis studies. Purified eosinophils were stimulated with HrHRF or PAF, and chemotaxis was assessed by using Boyden microchambers. In 3 experiments, the number of eosinophils migrating in the medium-control condition was 24 ± 4 . The number of eosinophils migrating in response to HrHRF (0.4 μ mol/L) was 54 ± 14 , or 225% of the medium-control value; to HrHRF (0.24 μ mol/L), 58 ± 6 , or 243%; and to HrHRF (0.12 μ mol/L), 18 ± 7 , which was not above the medium-control value. In comparison, the results with the 2 positive controls were that 473 ± 65 eosinophils (1971%) migrated in response to PAF (10^{-7} mol/L) and 157 ± 105 eosinophils (653%) migrated in response to RANTES (100 ng/mL).

HrHRF stimulates IL-8 production in the human eosinophilic cell line AML14-3D10

To demonstrate the effect of HrHRF on an eosinophilic cell line, AML14-3D10 cells were stimulated in the presence or absence of GM-CSF. Results were similar to those achieved with human eosinophils in the presence of GM-CSF: HrHRF stimulated IL-8 production to a level 2224 pg/mL above that with medium alone. Unlike eosinophils, AML14-3D10 cells did not need priming with GM-CSF for this to occur. This is not surprising, since these cells produce and use GM-CSF in an autocrine fashion.⁴⁴ Even in the

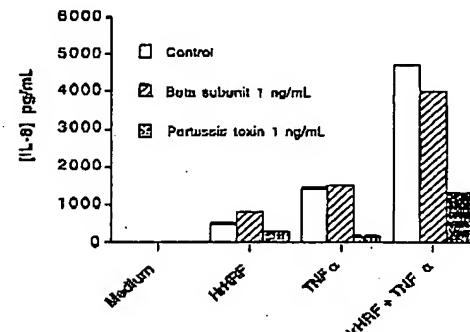


Figure 5. Pertussis toxin inhibits IL-8 secretion by eosinophils. Cells were stimulated with TNF- α (10 ng/mL), HrHRF (0.7 μ mol/L), or both in the absence (open bars) or presence of 1 ng/mL of either PT (closed bars) or its inactive β -oligomer (stippled bars). Data are the mean values from 2 experiments.

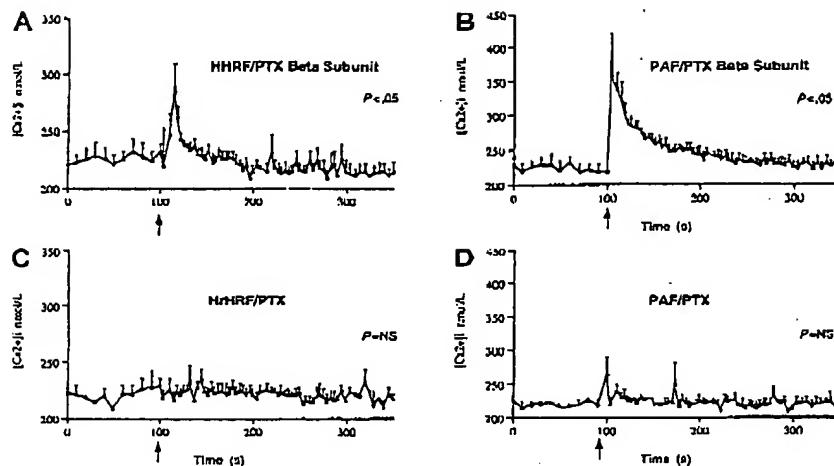


Figure 6. Changes in cytosolic free calcium in response to HrHRF or a known positive stimulus, PAF. Eosinophils were preincubated with either the inactive β -oligomer of PT (panels A,B) or PT (panels C,D) and loaded with Fura 2-AM. Cells were then stimulated (arrows) with $0.7 \mu\text{mol/L}$ HrHRF (panels A,C) or $1 \mu\text{mol/L}$ PAF (panels B,D). The average calcium response \pm SEM (vertical bars) of eosinophils from 4 donors is expressed as the relative change in Fura 2-AM saturation. P values indicate differences between 8 time points before and after the addition of stimulus.

absence of GM-CSF, HrHRF produced a level of IL-8 in the AML14-3D10 cell line that was 1217 pg/mL above that with the medium control ($n = 3$).

The effect of HrHRF on eosinophils and AML14-3D10 cells is not dependent on IgE

It was previously reported that human eosinophils do not express Fc ϵ RI α but do have an intracellular pool of IgE.¹⁷ We reproduced these data: our flow cytometry studies detected no extracellular expression of IgE, Fc ϵ RI α , or Fc ϵ RII on either human eosinophils or AML14-3D10 cells (data not shown). We next investigated whether the AML14-3D10 cells, like human eosinophils, had an intracellular pool of Fc ϵ RI α . Western blot analysis of AML14-3D10 cell lysates incubated with a mAb specific for Fc ϵ RI α showed no band, whereas the matched, positive-control basophil lysates had a distinct band of approximately 50 kd (Figure 7). Therefore, unlike human eosinophils, AML14-3D10 cells have no detectable Fc ϵ RI α . Thus, HrHRF activates eosinophils and AML14-3D10 cells through a mechanism that does not depend on IgE or the Fc ϵ RI receptor.

Discussion

The principal findings of this study are that HrHRF can activate a cell type other than the basophil and that it acts like a cytokine. Our experiments demonstrated that HrHRF causes IL-8 production from eosinophils and AML14-3D10 cells primed with GM-CSF and from AML14-3D10 cells in the absence of GM-CSF. Additionally, HrHRF is chemotactic for eosinophils in vitro in the absence of GM-CSF, although it is not known whether the concentrations required for chemotaxis have *in vivo* relevance. HrHRF also induced Ca^{++} mobilization in a subset of allergic donors in the absence of GM-CSF, although there was no difference in viability or cell number in cultures incubated with GM-CSF, we believe that GM-CSF may provide an activation signal allowing cells to respond to HrHRF, TNF- α , or both. Only the combination of TNF- α and IL-5 induced significant IL-8 production in eosinophils not primed with GM-CSF. However, even in this case, priming with GM-CSF greatly enhanced the magnitude of the response.

The necessity of GM-CSF for IL-8 secretion from eosinophils

stimulated by other stimuli, such as RANTES or PAF, was reported previously.²⁴ In contrast, Nakajima et al²³ showed that preincubation with GM-CSF was not required for IL-8 secretion from eosinophils when 25 ng/mL TNF- α was the stimulus. Although slightly different experimental conditions were used, one major difference between their study and ours was the inability of TNF- α alone to induce IL-8 secretion from GM-CSF-primed eosinophils. The reason for this difference is unclear, although it might be explained by the source of the eosinophils. We routinely used eosinophils from mildly allergic donors, whereas the cells used by Nakajima et al were primarily from healthy donors. Of note, Nakajima et al²³ also reported a significant enhancement by IL-5 of IL-8 induced by immobilized immunoglobulin, as well as a nonsignificant tendency of IL-5 to enhance TNF- α -induced IL-8 release. This parallels the results with the combination of HrHRF and TNF- α or TNF- α and IL-5 in our experiments.

In addressing the specificity of HrHRF activation of eosinophils, we also considered the following. Human eosinophils express Mac-1 (CD11b),⁴⁵ a molecule capable of binding various ligands, including LPS.⁴⁶ Our finding of no difference in IL-8 production between experiments using preparations containing barely detectable levels of endotoxin and experiments using preparations with high endotoxin levels rules out endotoxin as the stimulus for the results observed. Additionally, although both neutrophils⁴⁷ and monocytes⁴⁸ were previously shown to secrete IL-8 on stimulation with TNF- α , it is unlikely that these cells were responsible for the IL-8 production in our eosinophil preparations.



Figure 7. Western blot of cell lysates incubated with monoclonal antibody to the Fc ϵ RI α chain. For this experiment, 6.5×10^6 basophils or AML14-3D10 cells were lysed, and 500 μg cell lysate per lane were loaded on a gel, electrophoresed, and assessed by Western blot analysis. Basophils have a band of approximately 50 kd that corresponds to Fc ϵ RI α ; this is absent in the AML14-3D10 cells. Results shown are representative of 3 experiments.

Although eosinophils with greater than 98% purity were used in all our experiments, purity was essentially 100% in some experiments, and no differences in IL-8 production were observed.

It might be argued that IL-8 production by HRF that requires GM-CSF and TNF- α is only an in vitro phenomenon. However, HRF has been found in nasal lavages obtained during antigen-induced LPR⁴⁹ and in fluids from skin blisters.⁵⁰ Additionally, GM-CSF protein was a predominant cytokine in skin-blister fluids associated with LPR after an antigen challenge,⁵¹ and levels of messenger RNA (mRNA) for GM-CSF were significantly elevated in the bronchial mucosa of asthmatic subjects compared with healthy subjects.⁵² Furthermore, activated T cells⁵³ and eosinophils in vivo⁵⁴ have been reported as sources of GM-CSF. Although it remains unclear which signals induce GM-CSF production in eosinophils, it was shown that eosinophils from BAL fluids, but not from peripheral blood, have mRNA for GM-CSF.⁵⁴ In antigen-challenged lungs, TNF- α can be released from mononuclear cells stimulated with IgE and antigen complexes⁵⁵ and possibly from activated mast cells.⁵⁶ Indeed, TNF- α was found at significantly higher concentrations in BAL fluids and sputum of symptomatic allergic subjects.^{57,58} Therefore, we speculate that after exposure to an antigen, the appropriate cytokine environment would be available for enhancement of IL-8 production by HRF.

HrHRF induced increases in intracytoplasmic Ca⁺⁺ that were completely ablated by preincubation of eosinophils with PT. However, the Ca⁺⁺ responses induced by HrHRF were observed in only 25% of donors. The reason for this low response rate is unclear. It may have been a consequence of variability among different eosinophil preparations. It is unlikely that it was due to differences in expression of a putative HRF receptor because HrHRF always had an effect on cytokine production that was independent of Ca⁺⁺ mobilization.

It is intriguing that both secretion and Ca⁺⁺ mobilization in eosinophils induced by HrHRF were inhibited by PT, whereas the HrHRF-induced histamine release from basophils from IgE⁺ donors was not inhibited by PT (data not shown). One possible explanation for these findings is that there may be differential HrHRF signaling in these 2 cell types. There is precedent for this. The CC chemokine, RANTES, showed 2 types of Ca⁺⁺ signaling in T-cell lines.⁵⁹ The initial transient peak was sensitive to PT, whereas the second, delayed Ca⁺⁺ peak was inhibited by protein

tyrosine kinase inhibitors. These different Ca⁺⁺ responses are associated with different functional responses. Although one could propose that this difference is due to the presence of 2 different HrHRF receptors, as is the case with chemokine receptors,⁵⁹ another likely explanation is the use of different guanine nucleotide binding proteins (G proteins) in HrHRF signaling in these 2 cell types. Studies of cardiac phenomena and of bradykinins have found that different types of cells express different amounts of G proteins.⁶⁰⁻⁶² Additionally, the CC chemokine MIP-1 α is known to be coupled to multiple heterotrimeric G proteins—G(s), G(o), and G(z)—but not G(i).⁶³ These differences in expression of G proteins account for different effector functions and are being investigated.

Another difference between HrHRF activation of basophils and HrHRF activation of eosinophils was observed in the HrHRF priming experiments. Priming of basophils with HrHRF for 15 minutes and subsequent washing of the cells did not diminish histamine release (data not shown). This suggests a high-affinity interaction between HrHRF and the basophil. On the other hand, preincubation of eosinophils with HrHRF for 1 hour and washing abolished the enhancement of TNF- α -induced production of IL-8. These findings might appear contradictory, but they could be due to the time needed to generate the response in the 2 cell types. Histamine release is essentially complete within 15 minutes, whereas augmentation of IL-8 production takes more than 5 hours.

Eosinophils from selected patients with hypereosinophilia¹⁵ and eosinophils obtained after antigen challenge¹⁶ were reported to express Fc ϵ RI. However, these results are contradicted by the data of Seminario et al¹⁷ and Kita et al.¹⁸ Additionally, our data demonstrating HrHRF activation of AML14-3D10 cells, which do not possess Fc ϵ RI α , suggest that neither IgE nor an IgE-binding structure played a role in Ca⁺⁺ mobilization or regulation of cytokine production by HrHRF in our donors. Hence, we conclude that HrHRF contributes to eosinophil activation in a manner independent of Fc ϵ RI. Research is currently focused on molecular identification of the HRF-binding structure on the surface of inflammatory cells.

Acknowledgment

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